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Original article

Exploring the Therapeutic Efficiency of the Synergetic Role of Omega-3 and Vitamin E in Propionic Acid-Induced Autistic Features in Juvenile Rat Model

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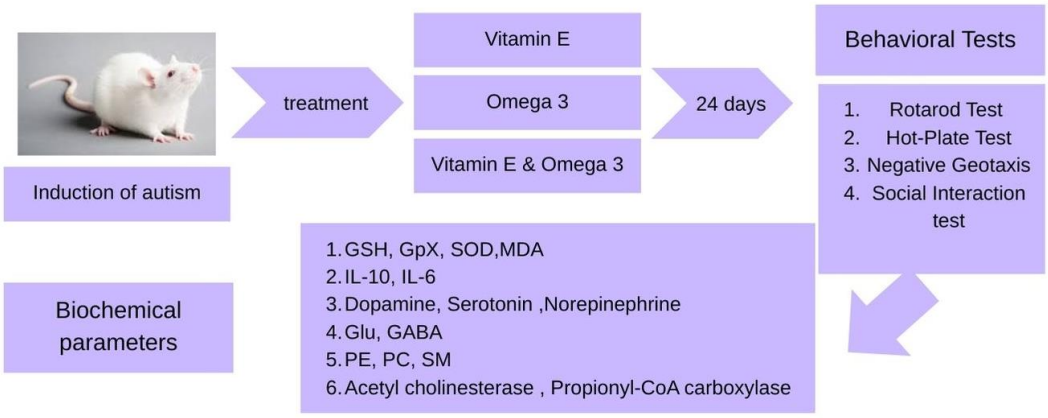
ABSTRACT

Autistic spectrum disorders (ASD) are neurodevelopmental disorders that affect behavior, communication, and social interaction. Over the past three decades, the prevalence of autism has increased at an alarming rate, though it varies by country. The present study aimed to evaluate the therapeutic benefits of vitamin E and. Omega-3 against propionic acid (PPA) induced autism in the rodent model. Forty male Wistar albino juvenile rats were divided equally into 5 groups. *Group 1*: received saline and served as control. *Group 2*: orally administered PPA at a dose of 250 mg /kg bw/day for 3 days and served as a positive control. Group 3: PPA was administered with vitamin E. Group 4: PPA was administered with omega-3. *Group 5*: PPA was administered along with vitamin E and omega-3. Behavioral patterns were observed, and oxidative stress-related markers were determined. Moreover, serotonin, dopamine, gamma-aminobutyric acid (GABA), glutamate, interleukins (IL-6 and IL-10), in addition to brain phospholipid profile as phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylcholine (PC) were measured. Acetylcholinesterase (AchE) and propionyl-CoA carboxylase (PCCA) activity were evaluated in all groups. PPA administration significantly reduced social performance, IL-10, dopamine, GABA, and phospholipid profile levels. It also increased oxidative stress biomarkers, IL-6, serotonin, and glutamate. Vitamin E and omega-3 supplementation showed potential in managing ASD by counteracting PPA-induced autistic features through their antioxidant and anti-inflammatory properties. In conclusion, our research suggests that vitamin E and omega-3 can counteract PPA-induced autistic features, highlighting their potential as therapeutic agents for ASD management.

Graphical abstract



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1. Introduction

Autistic spectrum disorders (ASD) are neurodevelopmental disorders that affect behavior, communication, and social interaction [1]. ASD is characterized by restricted and repetitive behaviors, along with social-communication difficulties. It is also known as Asperger's syndrome. The core features of ASD are represented by these two behavioral dimensions in the most recent conceptualization. ASD's diversity can be illustrated using related factors like cognitive and language ability [2]. Reports indicate an increase in ASD prevalence, with significant variations among different countries [3]. According to the Global Burden of Disease Study 2019, the age-standardized prevalence rate (ASPR) of ASD in North Africa and the Middle East was approximately 304.4 per 100,000 individuals in 2019. The incidence rate was 7.7 per 100,000, with a notable male predominance (ASPR 2.9 times higher in males) [4, 5]. Due to the unknown etiology and pathogenesis of ASD, there is no clinical biomarker [6]. The pathophysiology of ASD may involve mitochondrial dysfunction, immune dysregulation, oxidative stress, inflammation, and excitotoxicity [7]. Elevated oxidant levels lead to oxidative stress (OS), causing cellular damage and disrupting signal transmission and gene regulation [8, 9]. The brain is particularly vulnerable to OS due to its high energy requirement, limited antioxidant capacity, higher amounts of lipids, mainly PUFAs, and transition metals, e.g., iron and copper [10]. Studies have reported mitochondrial dysfunction and glutathione depletion in the brains of children with ASD [11].

Propionic acid (PPA), also known as methyl acetic acid and commercially as the food additive E280, is a short-chain fatty acid (SCFA) [12]. Along with other SCFAs, such as butyrate and acetate. These SCFAs, including butyrate and acetate, are primarily produced by fermentation of dietary carbohydrates and specific amino acids by gut microbiota [12]. PPA can cross the blood-brain and gut-blood barriers, allowing it to access the central nervous system [13].

Inside cells, PPA buildup causes intracellular acidification, which can alter neurotransmitter release, block gap junctions, and increase intracellular calcium release, leading to behavioral and neural transmission issues [14]. Furthermore, "High levels of PPA are often associated with oxidative stress, developmental delays, and immune or metabolic disturbances similar to those seen in autism [15]. Additionally, gut microbiota that produces PPA, such as *Bacteroidetes*, *Clostridia*, and *Desulfovibrio* genera, are frequently found in higher abundance in the guts of ASD patients [16].

Omega-3 plays a crucial role during pregnancy [17] and supports cardiovascular health [18]. Its anti-inflammatory properties make it helpful in treating inflammatory diseases like rheumatoid arthritis [19] and inflammatory bowel disease [20]. PUFA-enriched foods have been shown to reduce the risk of neurological abnormalities [21].

Moreover, Mobarakeh et al. [22] observed significantly low levels of vitamin E in children with ASD. Low blood levels of vitamin E have been linked to ASD-like behaviors [23]. Similarly, Alinaghi Langari et al. [24] demonstrated that administering vitamin E to rats exposed to valproic acid (VPA) during pregnancy significantly reduced repetitive and stereotyped behaviors, social deficits, and anxiety-like symptoms. Therefore, our study aimed to evaluate the therapeutic benefits of vitamin E and omega-3, individually and in combination, in ameliorating autism in a rat model.

2. Materials and Methods:

2.1. Materials

Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA) was the supplier of propionic acid (Cat. No. 79-09-4) and reagents for the HPLC kits. Vitamin E (1000 mg) was purchased from Pharmaco Pharmaceutical, Cairo, Egypt. Omega-3 (1000mg) capsules were purchased from Sedico Pharmaceutical, Cairo, Egypt.

2.2. Animal

All national, international, and institutional guidelines for animal care and use were followed. The Medical Research Ethics Committee (MREC) at the National Research Center, Dokki, Giza, Egypt, approved the protocol (Approval No. 1244052021). Procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). Forty male Wistar albino juvenile rats (3–4 weeks old, 31–45 g) were obtained from the animal house of the National Research Center, Dokki, Giza, Egypt. They were fed a standard diet supplied by El-Nasr Pharmaceutical Company, Egypt, containing: 72.2% carbohydrates, 3.4% fats, 19.8% proteins, 3.65% cellulose, 0.5% vitamins, minerals, and 0.5% salts.

2.3. Experimental design

Autism was induced by administering buffered PPA (250 mg/kg b.w/day) for 3 days, according to Alfawaz et al. [25]. The experiment continued for 30 days. Forty male Wistar albino juvenile rats were divided into five groups (8 rats/group) as follows: Group 1 received saline and served as a control. Group 2 received buffered PPA at 250 mg/kg b.w/day for three consecutive days. Group 3 received PPA and vitamin E at 100 mg/kg b.w in 1 ml corn oil for 30 days [26]. Group 4 received PPA and omega-3 at 100 mg/kg b.w/day for 30 days [27]. Group 5 received PPA and a combination of vitamin E and omega-3.

2.4. Behavioral tests

All behavioral tests were conducted between the 24th and 26th day of propionic acid injection.

2.4.1. Rotarod test

On the 24th and 26th days after PPA injection, each animal was placed on a rotating rod at 40 revolutions per minute (rpm). The endurance time was recorded as the duration each animal remained balanced on the rod for up to five minutes [28].

2.4.2. Hot-Plate test

According to Turner's method, the pain response to thermal stimuli was assessed between the 24th and 26th

day after propionic acid injection using a hot plate test apparatus **(Ugo Basile, Comerio, Italy).** Decreased pain sensitivity is a common feature of autism [29,30]. The latency of withdrawal response to heat stimulation was recorded and used as an indicator of pain sensitivity.

2.4.3. Negative Geotaxis

Negative geotaxis was assessed on the 24th and 26th days after the propionic acid injection by positioning the experimental rats on a 45° slope with their heads pointed downward. The time taken to rotate 180 degrees was recorded [31].

2.4.4. Social interaction

The impact of interventions on social behavior was assessed using Crawley's sociability and preference for social novelty protocol [32, 33]. This test measures the time spent with a novel conspecific and the preference for a novel versus a familiar conspecific to quantify social inclinations. The animals were housed separately the night before the experiment to enhance social connections. The primary objectives are to assess a) the amount of time spent with a novel conspecific and b) the preference for a novel versus a familiar conspecific to quantify the social inclinations of the experimental rats. The animals were housed separately the night before the experiment to enhance social connections.

2.5. Preparation of blood sampling:

At the end of the 30-day experiment, the animals were fasted for 12–14 hours. Blood samples were taken in clean, dry, sterilized centrifuge tubes under light diethyl ether anesthesia. The samples were allowed to clot and then centrifuged for 10 minutes at 4 °C at 3000 rpm to obtain serum, which was stored at -20 °C for biochemical analysis.

2.6. Brain tissue sampling:

After blood collection, rats were sacrificed by decapitation. The brain tissue was rapidly removed, perfused with ice-cold isotonic saline to remove blood clots, blotted on filter paper, weighed, and divided into two halves. The tissue was immediately stored at -20 °C for biochemical analyses.

2.7. Biochemical analysis:

Oxidative stress biomarkers were measured in brain tissue using commercial assay kits for malondialdehyde (MDA; CAT# NWK-MDA01, Biodiagnostic, Egypt), superoxide dismutase (SOD; CAT#SD2521, Biodiagnostic, Egypt), reduced glutathione (GSH; Cat. No. GR2511, Biodiagnostic, Egypt), and glutathione peroxidase (GPx; Cat. No. GP2524, Biodiagnostic, Egypt).

2.7.1. Determination of inflammatory mediators in brain tissue homogenate

Interleukin 6 (IL-6) was measured in brain tissue using the Rat IL-6 Immunoassay kit (Cat. No. R6000B) (R&D Systems, Minneapolis, MN, USA). Interleukin 10 (IL-10) was also estimated in brain tissue using an ELISA kit (Cat. No. MBS764911). Absorbance was measured at 450 nm following the manufacturer's instructions.

2.7.2. Determination of brain monoamines

Norepinephrine, dopamine, and serotonin levels were measured in brain tissue homogenate samples using an Agilent Technologies 1100 series (Santa Clara, California, USA) HPLC system with a quaternary pump (G131A model). As directed by the manufacturer, an ELISA assay kit (My BioSource, USA) (Cat No. MBS269152) was used to detect the amount of gamma-aminobutyric acid (GABA) in brain cells. Glutamate (GLU) levels were also measured in brain homogenate using an ELISA test kit (Cat. No. MBS756400) provided by MyBioSource (USA) according to the manufacturer's instructions. Also, the acetylcholinesterase (AChE) level was estimated using an ELISA Kit (CUSABIO, Houston, TX, USA) (Cat. No. CSB-E11304r) according to the manufacturer's instructions.

2.7.3. Determination of plasma phospholipid fractions

High-performance liquid chromatography (HPLC) was used to determine phospholipids, using an Agilent HPLC system 1100 series (Santa Clara, California, USA). Acetonitrile, methanol, and phosphoric acid (85%) with a ratio of 1000:40:0.4 v/v served as the mobile phase. It was given to the column at room temperature (25 °C) with a 1.5 mL/min flow rate. The UV-visible photodiode array detector's wavelength setting was 203 nm. Each sample was injected with 20 µL after dissolution in 2 mL of 2-propanol/ n-hexane (1:3 v/v). The resulting concentration was calculated using Agilent ChemStation software for LC and LC/MC systems (Agilent Technologies) [34, 35].

2.7.4. Determination of Propionyl-CoA Carboxylase (PCCA) level in brain tissue:

Following the manufacturer's instructions, the propionyl-CoA Carboxylase (PCCA) level was measured in brain tissue using an ELISA assay kit (MyBioSource, USA; Cat No. MBS9334641).

2.8. Statistical analysis

Data were analyzed using SPSS (Statistical Package for the Social Sciences) version 20 software package (USA). Results were expressed as mean ± S.D of 8 rats in each group. Statistical analysis was carried out by one-way analysis of variance (ANOVA), combined with the *Costat* software computer program, where unshared letters are significant at P value ≤ 0.05.

3. Results

3.1. Behavioral tests

3.1.1 Rotarod Test (indicators of motor activity, physical ability, balance, and coordination capability)

The PPA-treated male group exhibited a significant decrease in falling time compared to the control male group, with a 48.7% reduction (282.83s vs 145.00s, $p<0.001$). These results suggest that PPA treatment significantly impairs motor activity, physical ability, balance, and coordination (**Fig.1.A**).

3.1.2 Hot Plate Latency Test (Indicator of the pain response to thermal stimuli):

The PPA-treated males demonstrated a significant 51.3% reduction in withdrawal response latency com-

pared to control males (23.83s *vs* 11.60s, $p<0.0001$). This indicates that PPA treatment affects pain response to thermal stimuli, potentially increasing pain sensitivity (**Fig.1.B**).

3.1.3 Negative Geotaxis Test (placing the animal on a 45∘ slope with its head pointing down the decline):

PPA-treated male exhibited significant decreases in the time taken to turn 180° compared to their respective controls. PPA-treated males showed a 64.0% reduction (4.17s *vs* 1.50s, $p<0.0001$). These results suggest that PPA treatment enhances the negative geotaxis response in intoxicated rats (**Fig.1.C**).

3.1.4 Social Interaction Test (Indicators of the impairment of social interaction):

In the empty social chamber, PPA-treated rats spent 61.2% less time than normal rats (30.50s *vs* 11.83s, $p<0.05$). For interactions with familiar rats, PPA-treated rats spent 14.8% less time compared to controls (5.67s vs 4.83s, not significant). While with unfamiliar rats, PPA-treated rats spent 92.5% less time compared to controls (20.00s vs 1.50s, $p<0.01$). The preference index for social chamber interactions decreased by 167.9% in PPA-treated rats (0.53 *vs* -0.36, $p<0.01$). These results indicate that PPA treatment significantly impairs social interaction (**Fig.1.D, E, F)**.

3.2. Oxidative stress-related markers

3.2.1. Brain malondialdehyde (MDA) level and superoxide dismutase (SOD) activity:

Brain MDA levels were significantly increased ($p<0.001$) in all studied groups except PPA+ vit. E and PPA+vit. E + omega-3 group, compared to the control group. Administration of omega-3 and vitamin E alone or as combined therapy to PPA-challenged rats showed a significant reduction ($p<0.001$ & $p<0.01$) in MDA level, compared to PPA-injected rats. Furthermore, vit. E alone or combined with omega 3 therapy was significantly superior to omega 3 alone in alleviating oxidative stress among the autistic rats with a % change of 5.63% and 5.09%, respectively. Compared to controls, a significant reduction ($p<0.0001$) was observed in GSH level and GPx activity among PPA-intoxicated rats. Regarding SOD enzyme activity was significantly decreased ($p<0.01$) among all experimental groups, compared to a control group. In contrast, all the treated groups showed a significant increase ($p<0.01$) in SOD enzyme activity, compared to PPA-rats, however, they were still significantly lower ($p<0.01$) than the control, table (1).

3.2.2 Reduced glutathione (GSH) level and glutathione peroxidase (GPx) activity:

. The autistic rats showed a significant elevation ($p<0.01$) in GSH level and GPx activity, compared to untreated rats. Both omega-3 and vit. E administration either alone or as combined therapy significantly increases ($p<0.001$) the GSH level and enhances GPx enzyme activity. Also, omega-3 supplementation was superior to vit. E in improving GSH levels and GPx activity with % change of 26.32 for GSH and 20.75% for GPx, respectively. While supplementation of combined therapy (vit. E+ omega-3) to autistic rats recorded marked improvement in GSH levels and GPx activity, compared to their levels in other groups (Table 1).

3.3. Neuroinflammatory-related markers:

3.3.1. Serum Interlukin-10 (IL-10):

All studied groups exhibited a significant reduction ($p<0.01$) in serum IL-10 levels, compared to the control. In contrast, all autistic rats showed elevated ($p<0.001$) serum IL-10 levels, compared to PPA-intoxicated rats. Administration of omega-3 alone or with vitamin E enhanced IL-10 levels. The combined therapy significantly increased serum IL-10 levels compared to PPA-challenged rats. However, levels remained significantly lower than the control group, with a percentage change of 10.03%. Also, the administration of omega-3 alone enhanced the level of IL-10 compared to the control group, while supplementation of vitamin E alone recorded the least improvement compared to the control group (Table 1).

3.3.2. Serum Interlukin-6 (IL-6):

As declared in Table 1, all experimental groups exhibited elevation ($p<0.0001$) in serum IL-6 compared to control, indicating a pronounced inflammatory response. Significant inhibition ($p<0.001$) was observed in serum IL-6 levels among the different therapeutic groups, compared to PPA-intoxicated rats, but still higher than the control. Omega-3 supplementation alone or combined with vitamin E showed the most effective reduction in IL-6 levels. The combined therapy achieved a marked decrease ($p<0.0001$) with a 42.86% change from PPA levels, though still significantly higher than controls ($p<0.05$). Omega-3 alone outperformed vitamin E, showing a 100.03% improvement, while vitamin E alone had the least effect, with only a 112.41% change compared to controls. The results highlight Omega-3's superior anti-inflammatory potential, particularly in combination with vitamin E.

3.4 Brain neurochemistry and neurotransmitters

3.4.1 Dopamine, Serotonin, and Norepinephrine levels in brain tissue

The study revealed a significant decrease in brain dopamine levels ($p<0.05$) in all PPA-treated groups compared to the control. However, Omega-3 or vitamin E treatment significantly increased dopamine levels compared to PPA-induced rats. The most significant improvement was recorded for combined therapy, with a 14.86% increase in dopamine level. Notably, Omega-3 alone was more effective than vitamin E in elevating dopamine.

Regarding serotonin and norepinephrine, both neurotransmitters were significantly elevated ($p<0.01$) in PPA-treated rats, indicating hyperserotonemia and noradrenergic activation. Therapeutic interventions with vitamin E, Omega-3, or both led to a notable reduction in these elevated levels. The combined treatment again proved most effective, producing a greater reduction in serotonin and norepinephrine than either treatment alone. (Table 2).

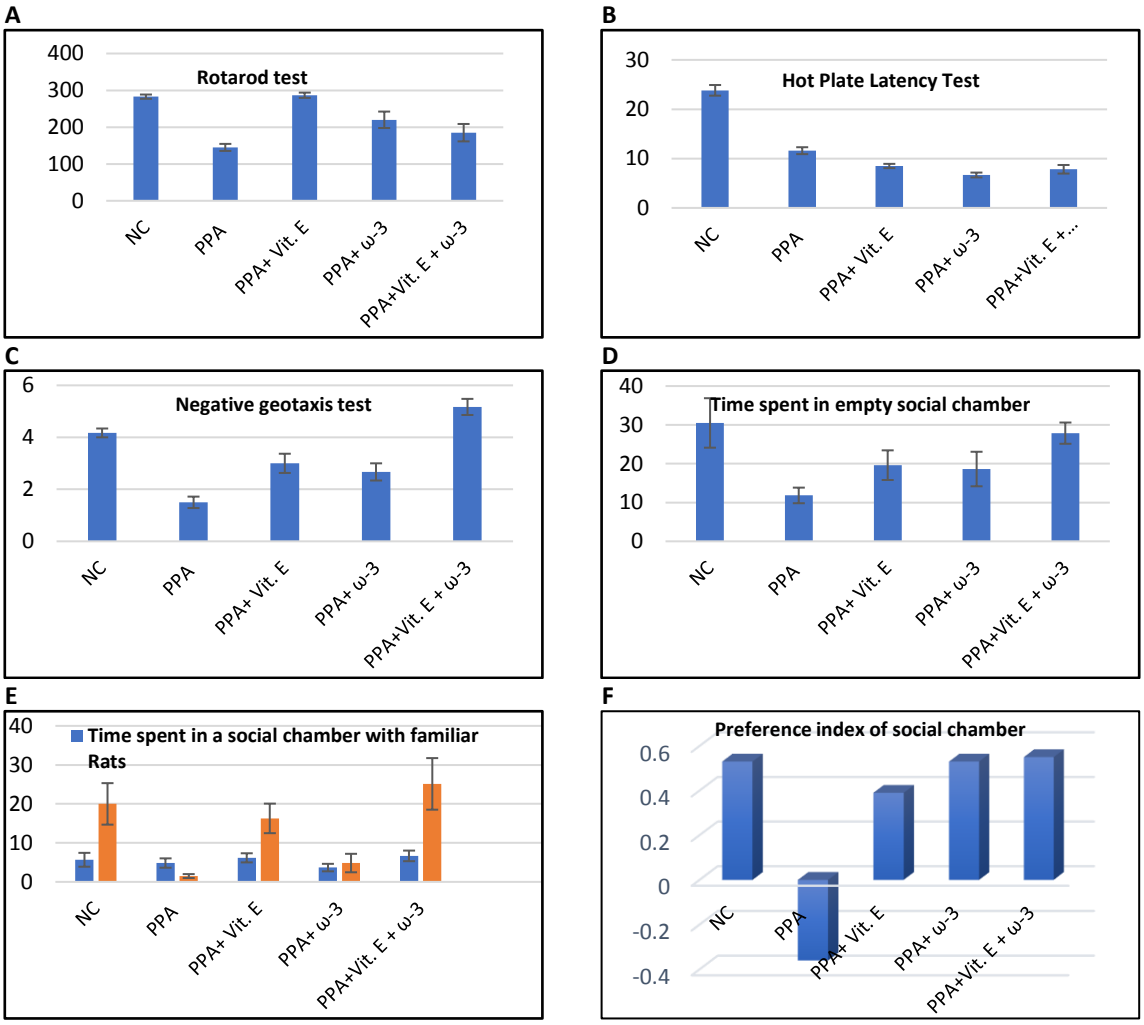

Fig.1: Behavioral tests among all experimental groups, data are expressed as Mean ± SD. NC: normal control; PPA: Propionic acid; Vit. E: vitamin E; ω-3: Omega-3. Least significant difference (LSD) at p<0.05. **(A)** Rotarod test, **(B)** Hot plate latency test, **(C)** Negative geotaxis test, **(D)** Time spent in empty social chamber, **(E)** Time spent in social chamber with familiar and unfamiliar rats, **(F)** Preference index of social chamber.

Table 1: Oxidative and inflammatory mediators in different experimental groups, data expressed as Mean±SD.

Parameters \ Groups	NC	PPA	PPA+ Vit. E	PPA+ ω-3	PPA+Vit. E + ω-3
MDA (nmol/g. tissue)	3.73±0.54^{c}	23.98 ±1.09^{a}	3.94 ±1.17^{c}	9.28 ±1.58^{b}	3.54 ±0.72^{c}
SOD (µmol/min/mg protein)	13.82 ± 2.45 ^{a}	8.18 ± 0.89 ^{d}	9.84 ± 1.52^{c}	9.62 ± 1.29^{c}	11.78 ± 0.79^{b}
GSH (mmol/ g. tissue)	89.66±6.39^{a}	42±7.47^{e}	50.88±1.80^{d}	66.06±1.38^{c}	72.74±2.45^{b}
GPx (U/gT)	48.61± 1.72 ^{a}	8.28 ± 0.80 ^{e}	14.95 ± 1.01^{d}	20.75± 0.95^{c}	38.5± 4.69^{b}
IL-10 (pg/ml)	153.57± 1.25^{a}	61.93± 1.68^{e}	111.1± 0.85^{d}	116.03± 0.95^{c}	138.16± 0.76^{b}
IL-6 (pg/ml)	36.56 ± 0.50^{e}	125.8± 0.4^{a}	77.66± 0.42 ^{b}	73.13± 0.65^{c}	52.23 ± 0.41 ^{d}

Data are presented as the mean ± standard deviation (SD), NC: normal control; PPA: Propionic acid; Vit. E: vitamin E; ω-3: Omega-3. MDA: Malondialdehyde; SOD: Superoxide dismutase, GSH: Reduced glutathione; GPx: Glutathione peroxidase. IL-10: interleukin-10; IL-6: interleukin-6. The same superscript letters mean non-significant. Least significant difference (LSD) at p<0.05.

3.4.2 Serum Glutamate (Glu) and Gamma amino butyric acid (GABA)

Data in Table 2 illustrate a significant elevation (p<0.001) in serum glutamate levels in all studied groups, compared to the control, treatment of PPA-injected male rats with vit. E and omega 3 only or combined improved the excitatory signals by reducing serum glutamate levels significantly (p<0.001), compared to untreated rats, while still higher than the control. The best results in glutamate reduction were recorded for combined therapy compared to either treatment alone.

Unlikely, serum gamma-aminobutyric acid (GABA) levels were decreased in all experimental groups, compared

to the control. Administration of both vitamin E and omega-3 only or as combined therapy improve the inhibitory neural signals by enhancing serum GABA levels significantly ($p<0.001$), compared to untreated autistic animals (Table 2).

Table 2: Neurochemistry, brain phospholipid profile, and neurotransmitters among the experimental groups.

Parameters \ Groups	NC	PPA	PPA+ Vit. E	PPA+ ω-3	PPA+Vit. E + ω-3
Dopamine (ng/g tissue)	4.91±0.03 ^{a}	2.58±0 .05^{e}	3.36± 0.01^{d}	3.68 ± 0.03^{c}	4.18± 0.03 ^{b}
Serotonin (µg/g tissue)	3.57± 0.25 ^{d}	6.2 ±0.26 ^{a}	4.58± 0.03 ^{b}	4.28± 0.07 ^{c}	4.08± 0.02 ^{c}
Norepinephrine(ng/gtissue)	2.86 ± 0.06^{e}	6.71 ± 0.1^{a}	3.65± 0.03^{b}	3.37 ± 0.02^{c}	3.16 ± 0.03^{d}
Glutamate (ng/ml)	36.36±0.37^{d}	118.33±0.35^{a}	63.40±0.65^{b}	71.63±0.45^{b}	53.33± 0.25^{c}
GABA (pg/ml)	18.53±0.25^{a}	6.17±0.14^{e}	12.13±0.15^{d}	12.8±0.2^{c}	15.36±0.25^{b}
Phosphatidylcholine (mg/dl)	40.63±0.61^{a}	2.23±0.30^{e}	12.63±0.21^{d}	30.26±0.05^{c}	33.56±0.35^{b}
Phosphatidylcholine (mg/dl)	18.01±0.22^{a}	1.2 ±0.05^{e}	4.67± 0.03^{d}	6.21± 0.08^{c}	9.21± 0.09^{b}
Sphingomyelin (mg/dl)	15.48±0.29^{a}	1.45± 0.22^{e}	6.66±0.15^{d}	7.62± 0.29^{c}	10.42±0.26^{b}
Acetylcholinesterase (pg/ml)	75.73±0.40^{e}	135.1±1.05^{a}	101.03±0.96^{b}	90.4±0.3^{c}	85.23±0.32^{d}
Propionyl-CoA carboxylase (ng/ml)	23.6 ±0.28^{a}	11.6 ±0.25^{e}	13.4 ±0.56^{d}	15.6 ±0.28^{c}	16.8 ± 0.14^{b}

Data presented as the mean ± standard deviation (SD), NC: normal control; PPA: Propionic acid; Vit. E: vitamin E; ω-3: Omega-3. GABA: Gamma-aminobutyric acid. The same superscript letters mean non-significant. Least significant difference (LSD) at $p<0.05$.

3.5. Brain phospholipid profile: phosphatidylethanolamine (PE), Phosphatidylcholine (PC), and Sphingomyelin (SM).

Autistic rats showed a highly significant reduction ($p<0.0001$) in serum levels of key phospholipids parameters; phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM) compared to the control-supplementation of vit. E and/or omega-3 improved the neurological functions and signal transduction by enhancing all phospholipids (PE, PC, and SM) levels significantly ($p<0.001$), compared to autistic PPA-induced rats. Similarly, combined therapy enhanced the PC levels in autistic rats significantly ($p<0.01$), with a % change of 48.86% for the treated groups (**Table** 2).

3.6. Acetylcholinesterase (AChE)

Marked elevation ($p<0.001$) was observed in serum AChE activity among the PPA-treated group, compared to the control group. Administration of omega-3 and/ or vitamin E. Improving the cholinergic neurotransmission by decreasing brain AChE activity. Significant inhibition ($p<0.05$) was observed in AChE enzyme activity in all therapeutic groups, compared to the PPA-autistic group. The superior effect was observed in autistic groups that received combined therapy (vit. E +omega-3), followed by omega-3 treated groups, then vit. E- Treated rats, compared to control groups (**Table** 2).

3.7. Propionyl-CoA carboxylase (PCC)

As shown in Table 2, autistic rats exhibit lower significant ($p<0.001$) brain propionyl-CoA carboxylase (PCC) enzyme activity, compared to the normal control group. As expected, the autistic group that received combined therapy (vitamin E with Omega-3) exhibited the best results in ameliorating PCC, compared to control groups.

4. Discussion

"Autism spectrum disorder is characterized by persistent challenges in social interaction, communication, and repetitive behaviors [36]. Although there is no known cure for ASD [37], vitamin E and omega-3 have been widely researched for their potential benefits.

Vitamin E is predominantly found in the phospholipid bilayer of cell membranes and is the most abundant lipophilic antioxidant in the brain. It protects polyunsaturated fatty acids (PUFA), other cell membrane components, and low-density lipoproteins from free-radical-mediated peroxidative damage [38].

Omega-3 fatty acids are PUFAs characterized by a methyl terminal group and a double bond in their molecular structure. Omega-3s play a crucial role in lipid metabolism and are abundantly found in nature, but cannot be synthesized by humans [39]. They protect brain cells against oxidative stress and inflammation, reducing the risk of neurological disorders [40].

In the current study, oral administration of PPA induced autistic features in rats. This result aligns with Erdogan et al. [41], who reported that PPA induces ASD-like symptoms similar to those in humans. Our research found that PPA treatment significantly reduced falling time, indicating impaired physical capacity, balance, coordination, and motor activity. These findings are consistent with Kamalmaz et al. [42], who observed motor deficits in PPA-treated mice, suggesting cerebellar dysfunction. Previous studies also link ASD to cerebellar damage and its connections to motor and prefrontal areas, contributing to poor coordination in both animals and humans [43]. Data from our study revealed that treating PPA-intoxicated rats with vitamin E improved their falling time, indicating better motor activity, physical ability, balance, and coordination. This effect is likely linked to vitamin E's role in regulating neurotransmitter systems, particularly dopamine. Studies have shown that developmental vitamin E deficiency is associated with increased anxiety, particularly in conditions like familial ataxia, and is linked to altered glutamate levels [44]. Similarly, Omega-3 fatty ac-

ids enhanced motor function in autistic rats. Xia et al. [45] found that Omega-3 reversed motor deficits in Parkinson's disease models by increasing latency time in Rotarod tests. Furthermore, Omega-3 helped reduce anxiety, neuronal damage, and dopaminergic deficits. In traumatic brain injury models, Omega-3 improved motor performance, reduced brain edema, and stabilized the blood-brain barrier by modulating apoptotic and anti-apoptotic pathways [46].

Pain sensitivity tests (hot plate test) showed that PPA-treated rats had decreased pain sensitivity, a typical ASD feature [47]. Vitamin E and Omega-3 treatments improved pain responses, suggesting neuroprotective effects [23, 48]. Regarding the negative geotaxis test, PPA treatment enhanced vestibular responses, whereas vitamin E and omega-3 improved locomotor function by stabilizing neural processes and promoting anti-inflammatory actions [49–51]. In the present study, the hot plate test, a widely used method for assessing pain response in rats, revealed that PPA-treated rats had significantly reduced withdrawal latency, suggesting decreased pain sensitivity, a common feature in autistic children [47]. This aligns with Podgorac et al. [52], who found delayed pain responses in VPA-exposed rats. Administration of vitamin E improved pain sensitivity in autistic rats, likely due to its role in correcting neurotransmitter imbalances and reducing oxidative stress [23]. Additionally, the current study showed that omega-3 supplementation to autistic rats caused a decrease in latency time and enhanced pain sensitivity, as supported by Unda et al. [48]. In the negative geotaxis test, PPA-treated rats showed faster 180° turning, indicating abnormal vestibular function. This effect is consistent with the results of Ruhela et al. [49]. Co-administration of vitamin E and Omega-3 increased the turning time, indicating restored motor coordination. Vitamin E's benefit is linked to its anti-cholinesterase properties [51], while omega-3 regulates neuroplasticity, and microglia activation, and reduces neuroinflammation. In the current study, combined administration of both supplements showed the most significant improvements, highlighting their synergistic neuroprotective effects.

In the current study, PPA treatment severely reduced sociability in rats, as shown by their preference for spending more time in an empty chamber and less time in a chamber with another rat, indicating poor social behavior. This was supported by Mirza & Sharma [53] and Hosny et al. [54], who also observed reduced social engagement in PPA-treated animals. However, vitamin E treatment improved social behavior, likely due to its ability to reduce stress-related hormones like corticosterone, as reported by Liang et al [55]. Similarly, omega-3 supplementation enhanced sociability in autistic rats, potentially through its neuroprotective effects, supporting brain cell communication and reducing neural damage. Our results are consistent with Madison et al. [56], who confirmed that omega-3 PUFAs can repair motor damage and have a protective role in an autism mouse model.

In ASD, elevated oxidative stress has been strongly linked to abnormal cellular processes, including lipid peroxidation, protein modification, and toxin accumulation [57, 58]. The central nervous system is particularly vulnerable to oxidative stress due to limited antioxidant penetration across the blood-brain barrier, such as vitamin E (alpha-tocopherol) [59]. One key marker of oxidative damage is malondialdehyde (MDA), a byproduct of lipid peroxidation that can damage proteins and DNA by forming protein adducts, potentially contributing to ASD pathophysiology [58–61].

In the present study, PPA-intoxicated rats exhibited a marked increase in MDA alongside with significant reduction in antioxidants (GSH, GPx, and SOD). These results align with previous studies by Ukkirapandian et al. [62], Zhang et al. [63], and Doğan et al. [64], who identified similar oxidative stress patterns in autism models and proposed low SOD as a potential ASD risk factor. Vitamin E supplementation significantly improved oxidative stress markers by lowering MDA and modulating GSH, GPx, and SOD levels confirming its antioxidant, anti-inflammatory, and neuroprotective properties [26, 65]. Likewise, Omega-3 treatment enhances intracellular antioxidant systems and regulates oxidative processes such as ferroptosis [66]. Clinical evidence from Doaei et al. [67] supports these findings, showing improved behavior and social communication in ASD children with ASD after Omega-3 supplementation.

Concerning immune system dysregulation, ASD children often exhibit immune system alterations, particularly in pro-inflammatory cytokine levels, IL-6, and anti-inflammatory cytokine IL-10 [68-70]. Cytokines can affect the CNS directly through different pathways, it can flow through the leaky areas of the blood-brain barrier (BBB), they can be actively transported by transport molecules that are unique to each cytokine, transmitted through cranial nerves and transferred peripherally activated monocytes can release cytokines which may disrupt neurodevelopment by altering synaptic processes and plasticity [71, 72]. As seen in the present study, oral administration of PPA to normal rats resulted in a considerable elevation in IL-6 and a decline in IL-10 levels, confirming a state of inflammation similar to that observed in ASD patients [53, 73]. However, there are obvious improvements in IL-6 and IL-10 upon treatment of PPA-autistic rats with vitamin E compared to untreated rats, suggesting its anti-inflammatory and neuroprotective effects [74, 75]. Similarly, the present results displayed that Omega-3 fatty acids administration enhanced IL-10 and reduced IL-6 levels, likely due to their anti-inflammatory, anti-apoptotic, and immune-modulating properties [76, 77]. Notably, the combined administration of vitamin E and Omega-3 resulted in greater improvements in cytokine regulation than either alone, confirming their synergistic effect in countering neuroinflammation caused by PPA. The current study demonstrates that PPA administration significantly disrupted neurotransmitter balance in the brain, a hallmark of ASD models. Specifically, do-

pamine (DA) levels were significantly reduced in all experimental groups exposed to PPA, while serotonin (5-HT) and norepinephrine (NE) levels were significantly elevated. These findings are consistent with earlier research showing that neuroinflammation and oxidative stress in ASD are associated with dopaminergic dysfunction and hyperserotonemia [25, 78].

Dopamine is responsible for social behavior, social communication, and movement control [79]. The observed reduction in DA levels among PPA-treated rats aligns with Alfawaz et al. [25], who reported that PPA administration led to a significant decline in dopamine due to neuroinflammatory and oxidative damage in dopaminergic neurons The study also showed that supplementation with omega-3 or vitamin E significantly elevated dopamine levels, with the combined therapy resulting in the highest improvement. These results are consistent with prior evidence showing that omega-3 FAs upregulate dopamine synthesis by enhancing tyrosine hydroxylase activity, the rate-limiting enzyme in dopamine production [80]. Vitamin E's neuroprotective effect also stems from its ability to neutralize oxidative radicals, protect dopaminergic neurons, and restore neurotransmitter balance [81]. The synergistic effect of co-administering omega-3 and vitamin E suggests a complementary mechanism: Omega-3 contributes to membrane fluidity and neurogenesis. In contrast, vitamin E provides antioxidant protection, crucial for dopaminergic neuronal health.

On the other hand, Serotonin (5-HT) plays a crucial role in mood regulation, social interaction, circadian rhythms, and sensory processing, while norepinephrine (NE) is involved in attention, stress response, and arousal [78,82,83]. In ASD, hyperserotonemia has been linked to abnormal emotional and social behavior [84]. The current results confirm this pattern. PPA-induced rats show significantly elevated serotonin and norepinephrine levels, a phenomenon supported by Ukkirapandian et al. [84], who linked increased 5-HT to impaired neurodevelopment and behavioral abnormalities. The elevation of NE may be due to enhanced sympathetic activity or neuroinflammatory stimulation of noradrenergic pathways. Treatment with Omega-3, vitamin E, or both significantly reduced 5-HT and NE levels, restoring them toward normal. The combined treatment again showed the most potent inhibitory effect, highlighting the enhanced efficacy of a multimodal therapeutic approach. These findings align with those of Chitre et al. [80], who showed that Omega-3 modulates monoaminergic systems, and with Rana et al. [81], who demonstrated that vitamin E reverses serotonin and norepinephrine dysregulation following brain injury. The current study supports the therapeutic potential of Omega-3 and vitamin E, both individually. Their administration restored dopamine levels and normalized the elevated serotonin and norepinephrine levels, reflecting a broad-spectrum neuroregulatory effect.

Glutamate and gamma-aminobutyric acid (GABA) are critical neurotransmitters that maintain the brain's excitatory-inhibitory balance. Glutamate facilitates excitatory transmission and supports brain development, learning, and memory, while GABA provides inhibitory regulation essential for controlling neural activity and behavior [85]. An imbalance between these systems can contribute to neurological disorders, including ASD. In the present study, PPA-induced autistic rats showed a significant elevation in glutamate and reduction in GABA, disrupting the normal neurotransmitter balance. These findings are consistent with the study of Alsubaiei et al. [86], suggesting that oxidative stress and inflammation impair neurotransmitter function by damaging mitochondria and altering cytokine signaling.

Vitamin E supplementation reverses this imbalance by decreasing glutamate levels and increasing GABA due to its antioxidant, anti-inflammatory, and neuroprotective properties. Similar results were reported by Abu-Elfotuh et al. [87], who observed restored GABA/glutamate ratios in Parkinsonism models. Likewise, Omega-3 administration significantly improved neurotransmitter balance. This effect is attributed to its role in neuroplasticity, reducing oxidative stress, and modulating neurotransmitter systems [88]. Our results align with Wattanathorn [89], who found improved GABA function and memory in rats given Omega-3-rich tuna oil. On the other hand, co-administration of both Omega-3 and vitamin E showed a marked increase in GABA level with depletion in glutamate level, which may be due to the synergistic effect of both.

Respecting phospholipids, phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM) are essential structural components of cell membranes, contributing to membrane fluidity, integrity, signaling, and cellular compartmentalization [90]. The current study's findings revealed a notable reduction in phospholipid levels (PE, PC, and SM) in PPA-autistic rats. These changes were addressed by Thomas et al. [91], who explained that the alterations in lipid metabolism influence membrane fluidity, peroxisomal activity, the ability for gap junctions to couple, signaling processes, and neuroinflammation, which could be linked to the development of ASD.

The administration of vitamin E to autistic rats resulted in a notable increase in phospholipid levels, reflecting its neuroprotective and antioxidant actions, including reducing oxidative stress, mitigation of mitochondrial dysfunction, excitotoxicity, and lipid peroxidation [92, 93]. Our findings align with Soto and his colleagues [93], who observed high phospholipid levels following vitamin E treatment in COVID-19 patients.

Comparable to vitamin E, Omega-3 supplementation restored phospholipid levels, particularly sphingomyelin, supporting its role in maintaining neuronal membrane fluidity and reducing neuroinflammation. This effect may also be linked to omega-3s' ability to inhibit sphingomyelinase enzymes, preventing sphingomyelin degradation [94]. Notably, combined administration of vitamin E and Omega-3 led to noticeable improvements in phospholipid content compared to either treatment alone. This synergis-

tic effect may be attributed to their complementary actions in preventing apoptosis, enhancing antioxidant defenses, and supporting neuronal health by regulating the expression of anti- and pro-apoptotic proteins [95].

Acetylcholine, a neurotransmitter within the cholinergic system, plays a significant role in memory functions. Research has explored the possibility that reduced levels of acetylcholine may contribute to the symptoms associated with autism [96, 97]. In the present study, PPA-induced rats significantly increased AChE activity, driven by oxidative stress. Vitamin E treatment significantly reduced AChE levels, enhancing synaptic acetylcholine and thus improving cognitive function and behavior. These effects are consistent with findings by Kandeil et al. [98], highlighting vitamin E's antioxidant, neuroprotective, and anti-anxiety roles. Similarly, Omega-3 supplementation inhibited AChE activity, as supported by Wattanathorn [89], who demonstrated its role in reducing oxidative stress and modulating inflammation. Additionally, combined therapy further reduced AChE activity, indicating synergistic neuroprotective effects. Our findings indicate that Omega-3 administration either alone or in conjunction with vitamin E led to a significant reduction in AChE activity. This effect may be attributed to the antioxidant, anticholinesterase, and anti-inflammatory properties of Omega-3 [99]. Additionally, the enzyme (PCC) facilitates the carboxylation of propionyl-CoA to D-methylmalonyl-CoA in the presence of ATP and is located in the mitochondria. It is also involved in metabolizing PPA, functionally linked to propionyl-CoA carboxylase in ASD. Dysfunction in PCC and accumulation of PA have been observed in ASD patients, providing a metabolic basis for the condition and validating the PPA rat model used in this study [100,101].

5. Conclusion

This comprehensive scientific and statistical analysis demonstrated that omega-3 supplementation has a beneficial impact, leading to improvements in autism-related characteristics such as stereotypical behaviors and social communication Furthermore, the combination of omega-3 with vitamin E amplified these effects and mitigated behavioral changes in rats with ASD These findings suggest that omega-3 and vitamin E, both individually and in combination, hold significant therapeutic potential for managing ASD symptoms.

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